

Critical Role for Mast Cells in Interleukin-1 β -Driven Skin Inflammation Associated with an Activating Mutation in the Nlrp3 Protein

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<http://dx.doi.org/10.1016/j.immuni.2012.04.013>

SUMMARY

Cryopyrin-associated periodic syndromes (CAPS) are caused by aberrant interleukin-1 β (IL-1 β) production induced by mutations in the NLRP3 protein in humans, but the mechanisms involved remain poorly understood. Using a mouse model, we show a role for the indigenous microbiota and mast cells (MCs) in skin disease associated with mutant Nlrp3 protein. Unlike normal cells, MCs expressing mutant Nlrp3 produced IL-1 β in response to lipopolysaccharide or tumor necrosis factor- α (TNF- α). In neonatal mice, the microbiota induced TNF- α and IL-1 β and promoted skin disease. MC deficiency greatly reduced disease in Nlrp3 mutant mice, and reconstitution of MC-deficient mice with mutant MCs restored skin disease, which required the expression of IL-1 β in MCs. Surprisingly, neutralization of TNF- α abrogated IL-1 β production and skin disease in neonatal Nlrp3 mutant mice, but not in affected adult mice. Thus, the microbiota and MCs initiate cellular events leading to dysregulated IL-1 β production and skin inflammation in neonatal mice with the CAPS-associated Nlrp3 mutation.

INTRODUCTION

The innate immune system is activated through the engagement of host soluble factors and germline-encoded pattern-recognition receptors (PRRs) by microbial moieties or endogenous molecules generated in the setting of infection or cellular injury (Chen and Núñez, 2010; Kawai and Akira, 2010). In response to infection, PRR activation initiates signal-transduction pathways that ultimately culminate in host defense responses that eliminate microbial invasion. A major inflammatory pathway is the inflammasome, a multiprotein platform that activates the protease caspase-1 (Franchi et al., 2009a; Schroder and Tschopp, 2010). Once activated, caspase-1 proteolytically

processes pro-interleukin-1 β (IL-1 β) and pro-IL-18, which is important for secretion of the biologically active forms of these cytokines (Chen and Núñez, 2010). To date, several inflammasomes have been described, of which three, the NLRP1, NLRP3, and NLRC4 inflammasomes, contain a PRR that belongs to the intracellular Nod-like receptor (NLR) family. Activation of the NLRP3 inflammasome is mediated by two signals. The first signal, referred to as priming, is the nuclear-factor- κ B-dependent transcription of pro-IL-1 β and NLRP3 through the stimulation of PRRs by various Toll-like receptor (TLR) agonists, including lipopolysaccharide (LPS), or certain cytokines such as tumor necrosis factor- α (TNF- α) or IL-1 β (Bauernfeind et al., 2009; Franchi et al., 2009b). The second signal activates NLRP3 and is induced by ATP, certain bacterial toxins, or a variety of crystalline and particulate matter, including urate crystals, asbestos fibers, silica, and aluminum salts (Franchi et al., 2010). In response to activating stimuli, NLRP3 recruits the adaptor protein ASC (apoptosis-associated speck-like protein containing a caspase recruitment and activation domain) and forms an inflammasome that drives caspase-1 activation (Franchi et al., 2009c).

The importance of the NLRP3 inflammasome is underscored by the observation that missense mutations in the *NLRP3* gene are responsible for a spectrum of autoinflammatory syndromes (Aksentijevich et al., 2002; Dowds et al., 2004; Hoffman et al., 2001). These diseases, collectively referred to as cryopyrin-associated periodic syndromes (CAPS), are rare monogenic inherited disorders that are characterized by episodes of fever, urticarial-like skin rash, and other more variable inflammatory manifestations, in the absence of autoimmunity or infection (Aksentijevich et al., 2007; Neven et al., 2004). CAPS include familial cold autoinflammatory syndrome (FCAS [MIM 120100]), Muckle-Wells syndrome (MWS [MIM 191900]), and neonatal-onset multisystem inflammatory disease (NOMID [MIM 607115]). Although CAPS are considered distinct clinical entities, they represent a continuum in disease severity; FCAS patients are the least affected, MWS patients are in the middle, and NOMID is the most severe form of disease, causing neurological deficits and deforming arthritis (Neven et al., 2004). These CAPS-associated missense *NLRP3* mutations result in enhanced activation of caspase-1 and secretion of IL-1 β by causing

constitutive activation of the NLRP3 inflammasome (Agostini et al., 2004). Notably, treatment of CAPS patients with an IL-1 receptor antagonist or IL-1 β -blocking antibody resolves most inflammatory signs and symptoms in CAPS, resulting in life-altering outcomes (Hoffman et al., 2004; Hoffman et al., 2008; Lachmann et al., 2009). Although CAPS are typically dominantly inherited disorders and exhibit high penetrance, there is evidence that environmental factors may play a role in triggering disease. For example, FCAS inflammatory episodes are often induced by systemic exposure to cold, MWS attacks can be induced by several triggers, and NOMID occurs at birth or in early infancy, but the environmental stimuli that induce disease onset remain unknown.

Two groups have recently generated gene-targeted mice harboring *Nlrp3* mutations that mimic the amino-acid substitutions found in CAPS (Brydges et al., 2009; Meng et al., 2009). Brydges et al. generated two disease-associated *Nlrp3* mouse strains; one line expressed A350V, corresponding to human A352V associated with MWS, and another line expressed L351P, corresponding to the FCAS-associated L353P mutation (Brydges et al., 2009). Practically all mice from the latter *Nlrp3* mutant strains die within 2 weeks after birth due to severe systemic inflammation (Brydges et al., 2009). In contrast, Meng et al. generated *Nlrp3* mutant mice that expressed *Nlrp3*^{R258W}, corresponding to the MWS-associated R260W NLRP3 mutation (Meng et al., 2009). Heterozygous *Nlrp3*^{R258W} mice exhibited delayed growth, reduced body weight, and increased mortality, but the great majority of *Nlrp3*^{R258W} mice survived after weaning (Meng et al., 2009). Notably, *Nlrp3* mutant mice from both groups developed dermatitis rich in neutrophils, which histologically resembles that observed in human CAPS (Brydges et al., 2009; Meng et al., 2009). Consistent with constitutive activation of the inflammasome, priming of macrophages from *Nlrp3* mutant mice with TLR agonists alone induced caspase-1 activation and robust IL-1 β production (Brydges et al., 2009; Meng et al., 2009). Furthermore, genetic deletion or neutralization of IL-1 signaling induced marked improvement of disease, whereas ablation of *Asc* abrogated perinatal mortality and cutaneous disease in *Nlrp3* mutant mice (Brydges et al., 2009; Meng et al., 2009).

Several hematopoietic cells, including macrophages, dendritic cells, neutrophils, and mast cells (MCs), can produce IL-1 β in response to microbial and endogenous stimuli (Franchi et al., 2007; Ghiringhelli et al., 2009; Nakamura et al., 2009). Surprisingly, most of the IL-1 β -positive cells in the skin lesions of CAPS patients are MCs (Nakamura et al., 2009). Furthermore, MCs can produce IL-1 β via the *Nlrp3* inflammasome (Nakamura et al., 2009). However, the role of MCs in skin disease associated with *Nlrp3* mutations has not been investigated. Additionally, the environmental and host factors that initiate IL-1 β -driven skin inflammation in the context of disease-associated *Nlrp3* mutations remain elusive. In the present study, we show that MCs play a critical role in triggering disease in neonatal *Nlrp3* mutant mice. Production of TNF- α and IL-1 β by MCs was important for IL-1 β -driven skin disease in *Nlrp3* mutant mice. Furthermore, we provide evidence that the indigenous microbiota promote TNF- α and IL-1 β production and contribute to skin disease in *Nlrp3* mutant mice.

RESULTS

Cutaneous Inflammation Induced by *Nlrp3*^{R258W} Mutation Requires MCs

Previous studies showed that the majority of IL-1 β -producing cells in the dermis of skin lesions from CAPS patients are MCs (Nakamura et al., 2009). Therefore, we tested whether MCs play a role in skin inflammation of mice expressing an R258W mutation in *Nlrp3*. To assess this, we crossed heterozygous *Nlrp3*^{R258W} mice with C57BL6-*Kit*^{W-sh/W-sh} mice to generate littermate mice expressing the *Nlrp3*^{R258W} mutation in the normal and MC-deficient background. Within the first week after birth, *Nlrp3*^{R258W} and wild-type littermates were indistinguishable. However, beginning 7 days after birth, *Nlrp3*^{R258W} mice raised under pathogen-free conditions displayed impaired weight gain (Figure 1A). In contrast, the increase in body weight was comparable in *Nlrp3*^{R258W}*Kit*^{W-sh/W-sh} and their control B6-*Kit*^{W-sh/W-sh} littermates (Figure 1A). Notably, neonatal *Nlrp3*^{R258W} mice developed elevated amounts of IL-1 β in serum and splenomegaly, which was greatly attenuated or abolished in their *Nlrp3*^{R258W}*Kit*^{W-sh/W-sh} littermates (Figures 1B and 1C). In addition, 100% of the *Nlrp3*^{R258W} mice developed skin disease in the posterior collar area and perianal region by 2 weeks after birth (Figures 1D and 1E; Figure S1 available online). Furthermore, the incidence and severity of skin disease in *Nlrp3*^{R258W}*Kit*^{W-sh/W-sh} mice was greatly reduced compared to their *Nlrp3*^{R258W} littermates (Figures 1D and 1E; Figure S1). Consistent with these findings, the skin of *Nlrp3*^{R258W} mice showed hyperkeratosis and marked neutrophil-rich infiltrate, which were absent or highly reduced in the same skin area of *Nlrp3*^{R258W}*Kit*^{W-sh/W-sh} mice (Figure 1F). As expected, abundant MCs were observed in the skin of *Nlrp3*^{R258W} mice, but not in neonatal *Nlrp3*^{R258W}*Kit*^{W-sh/W-sh} mice (Figure 1G). Immunohistochemistry revealed that cells labeled with avidin (a marker of MCs) produced IL-1 β in the dermis of neonatal *Nlrp3*^{R258W} mice, but not in their *Nlrp3*^{R258W}*Kit*^{W-sh/W-sh} or wild-type littermates (Figure 1G). These results indicate that MCs play a critical role in the development of cutaneous inflammation induced by the disease-associated *Nlrp3*^{R258W} mutation.

Priming with LPS or TNF- α Is Sufficient to Induce Caspase-1 Activation and IL-1 β Secretion in MCs Expressing the *Nlrp3*^{R258W} Mutation

To begin to understand how MCs contribute to inflammatory skin disease, we prepared bone marrow-derived MCs (BMCMCs) from wild-type and *Nlrp3*^{R258W} mice and determined their ability to produce IL-1 β through the *Nlrp3* inflammasome. BMCMCs from *Nlrp3*^{R258W} and wild-type mice exhibited comparable morphology, expression of CD117, and the ability to release β -hexosaminidase in response to several stimuli (Figure S2). As previously reported (Nakamura et al., 2009), IL-1 β secretion by MCs from wild-type mice required both priming with LPS and stimulation with ATP or the RNA-like molecule R837 (Figure 2A). In contrast, stimulation with LPS was sufficient to induce robust IL-1 β secretion in MCs from *Nlrp3*^{R258W} mice (Figure 2A). This differential response was specific in that MCs from wild-type and *Nlrp3*^{R258W} mice produced comparable amounts of TNF- α in response to the same stimuli (Figure 2A). Whereas stimulation with both LPS and ATP was required to induce processing of

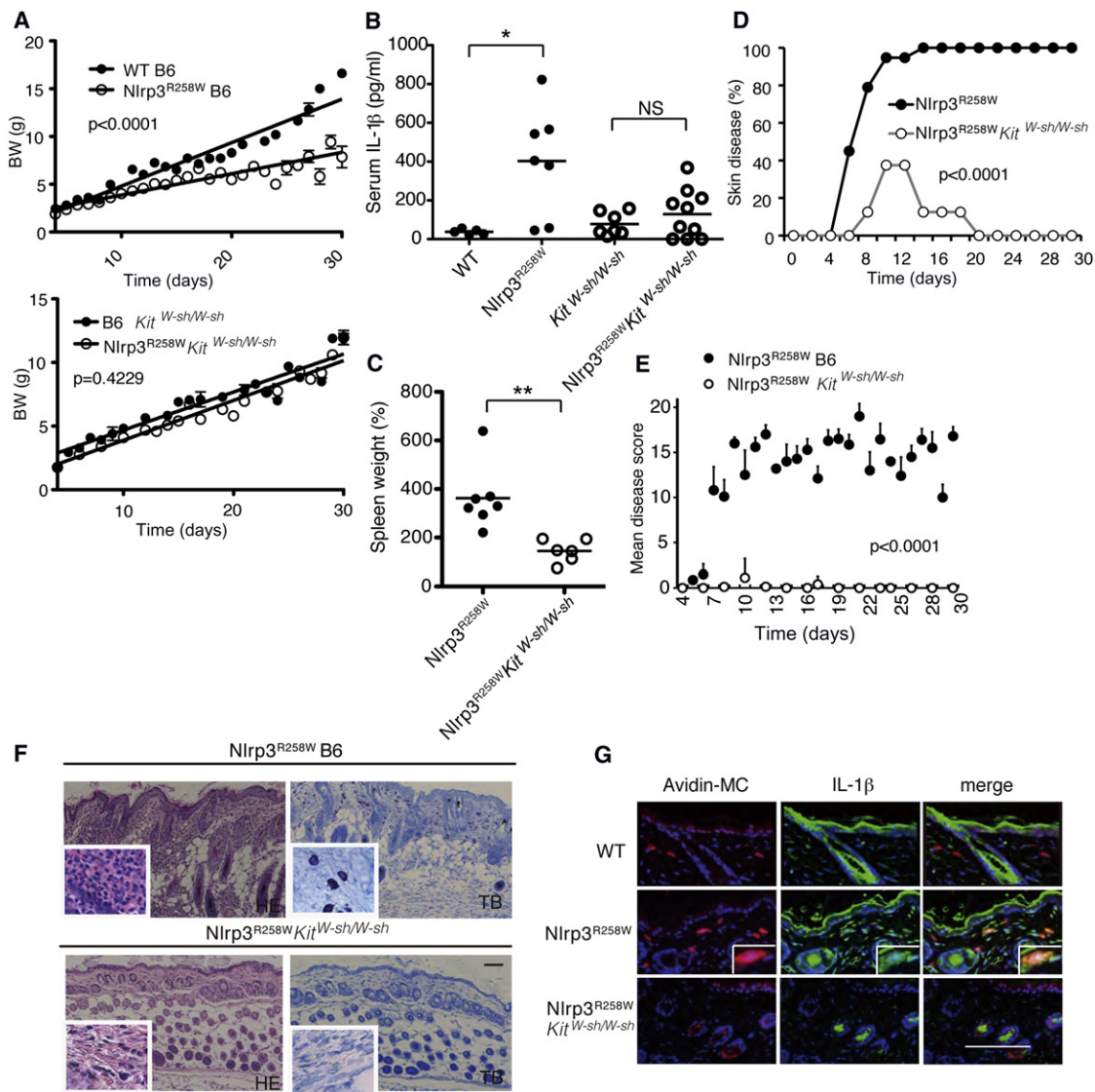


Figure 1. Cutaneous Inflammation in *Nlrp3*^{R258W} Mice Requires MCs

(A) Linear body weight (BW) curves of wild-type (WT, $n = 13$) and *Nlrp3*^{R258W} ($n = 20$) mice (left panel) and B6 *Kit*^{W-sh/W-sh} ($n = 10$) and *Nlrp3*^{R258W} *Kit*^{W-sh/W-sh} ($n = 10$) mice (right panel). Error bars represent mean \pm SEM.
 (B) Amounts of IL-1 β in serum of WT, *Nlrp3*^{R258W}, B6 *Kit*^{W-sh/W-sh}, and *Nlrp3*^{R258W} *Kit*^{W-sh/W-sh} mice.
 (C) Spleen weight of WT, *Nlrp3*^{R258W}, B6 *Kit*^{W-sh/W-sh}, and *Nlrp3*^{R258W} *Kit*^{W-sh/W-sh} mice.
 (D) Percentage of B6 *Kit*^{W-sh/W-sh} and *Nlrp3*^{R258W} *Kit*^{W-sh/W-sh} mice that developed skin disease in the neonatal period. Results are derived from mice depicted in (A).
 (E) Skin-disease score in *Nlrp3*^{R258W} and *Nlrp3*^{R258W} *Kit*^{W-sh/W-sh}. Results are derived from mice depicted in (A). Error bar indicates mean \pm SEM.
 (F) H&E (HE) and toluidine blue (TB) staining of involved skin from *Nlrp3*^{R258W} and comparable region of *Nlrp3*^{R258W} *Kit*^{W-sh/W-sh}.
 (G) Immunofluorescence staining of avidin-positive MCs (red) and IL-1 β (green) of involved skin from *Nlrp3*^{R258W} and comparable region from WT B6 and *Nlrp3*^{R258W} *Kit*^{W-sh/W-sh}. Merged images are also shown. Inset represents high magnification of avidin-positive MC producing IL-1 β (yellow-orange color). Notice autofluorescence of epidermis and hair follicle in WT and *Nlrp3*^{R258W} mice. Scale bar represents 100 μ m.

pro-caspase-1 into its active p20 subunit in wild-type MCs, stimulation with LPS was sufficient to activate caspase-1 in MCs from *Nlrp3*^{R258W} mice (Figure 2B). In addition, treatment with TNF- α alone induced IL-1 β secretion in MCs from *Nlrp3*^{R258W} mice, whereas both TNF- α and ATP were required to induce IL-1 β secretion in wild-type MCs (Figure 2C). Collectively, these results indicate that the *Nlrp3*^{R258W} protein is constitutively active in MCs in that IL-1 β production only requires a priming step.

IL-1 β Production by MCs Expressing the *Nlrp3*^{R258W} Mutation Is Important for Disease Development in Neonatal *Nlrp3*^{R258W} Mice

To determine whether IL-1 β production is important for disease development, we crossed heterozygous *Nlrp3*^{R258W} mice with *Il1b*^{-/-} mice to generate mice expressing the *Nlrp3*^{R258W} mutation in the presence and absence of IL-1 β . *Nlrp3*^{R258W} mice lacking IL-1 β developed normally after birth and, unlike their *Nlrp3*

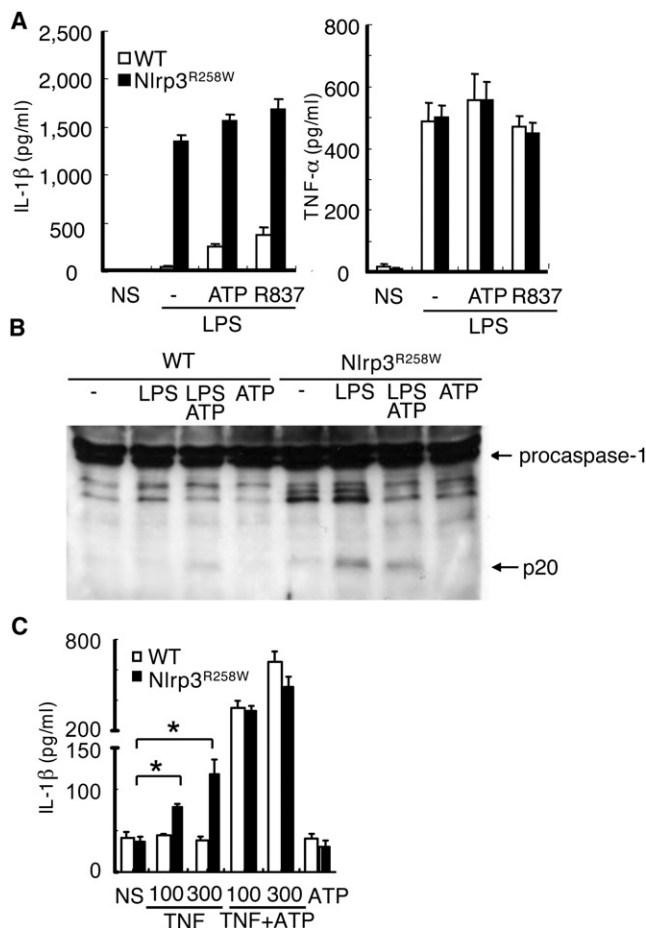


Figure 2. Priming with LPS or TNF- α Is Sufficient to Induce Caspase-1 Activation and IL-1 β Secretion in MCs Expressing the Nlrp3^{R258W} Mutation

(A) BMCMCs were incubated with LPS (100 ng/ml) for 15 hr and stimulated with ATP (5 mM) or R837 (100 μ M) for 30 min. IL-1 β (left) and TNF- α (right) in culture supernatants was measured by ELISA.

(B) Immunoblot analysis of extracts of cells together with cell supernatant of BMCMCs from Nlrp3^{R258W} and wild-type (WT) mice. Cells were incubated with LPS (100 ng/ml) for 4 hr and then stimulated by ATP (5 mM) or left untreated.

(C) BMCMCs were pretreated with TNF- α (100 ng/ml or 300 ng/ml) for 24 hr and stimulated with ATP (5 mM) for 30 min. IL-1 β in culture supernatants were measured by ELISA. Error bars represent mean \pm SD. Significance was determined by two-tailed Student's *t* test; **p* < 0.05. Data shown are representative of three independent experiments.

mutant littermates, they gained weight and did not develop skin disease (Figures 3A and 3B). To determine whether IL-1 β production by MCs is important for disease development, we adoptively transferred BMCMCs from wild-type, Nlrp3^{R258W}, or Nlrp3^{R258W}*Il1b*^{-/-} mice into the skin of B6-Kit^{W-sh/W-sh} or Nlrp3^{R258W}*Kit*^{W-sh/W-sh} mice at postneonatal day 1 (PND 1). MC-deficient recipient mice were reconstituted with wild-type or mutant MCs in the skin of the posterior collar area, where disease develops in 100% of neonatal Nlrp3^{R258W} mice. Neonatal B6-Kit^{W-sh/W-sh} mice reconstituted with MCs from wild-type, Nlrp3^{R258W}, or Nlrp3^{R258W}*Il1b*^{-/-} mice contained 3- to 4-fold more MCs than their B6-Kit^{W-sh/W-sh} littermates

and ~40% of conventional-type MCs present in the same skin area of age-matched wild-type or Nlrp3^{R258W} mice (Figure S3). Notably, Nlrp3^{R258W}*Kit*^{W-sh/W-sh} recipient mice adoptively transferred with MCs from Nlrp3^{R258W} mice, but not from Nlrp3^{R258W}*Il1b*^{-/-} or wild-type mice, developed inflammatory skin disease at the site of MC reconstitution that was comparable to that observed in nonmanipulated Nlrp3^{R258W} mice (Figure 3C; Figure S3E). Disease development was associated with reduced body weight (Figure 3D) and production of IL-1 β in the skin (Figure 3E). Notably, adoptive transfer of MCs from Nlrp3^{R258W} mice into B6-Kit^{W-sh/W-sh} mice did not induce skin disease (Figures 3C and 3E). These results indicate that MCs expressing the Nlrp3^{R258W} mutation and IL-1 β are important for disease, but also suggest that another cell harboring the Nlrp3^{R258W} mutation is also required for disease development.

Depletion of the Microbiota Inhibits Disease Development in Nlrp3^{R258W} Mice

The skin lesions observed in newborn Nlrp3^{R258W} mice developed in limited areas such as the posterior collar area and perianal region (Figure S1), suggesting that environmental stimuli may play a role in triggering cutaneous disease. Because microbial TLR ligands such as LPS are necessary and sufficient to induce robust IL-1 β production in MCs expressing the Nlrp3^{R258W} mutation (Figure 2A), we examined whether depletion of commensal bacteria with antibiotics could alter the development of skin disease. Newborn mice are colonized soon after birth by commensal bacteria derived from their mother (Hasegawa et al., 2010). Therefore, we treated pregnant female Nlrp3^{R258W} mice with a cocktail of antibiotics in the drinking water for 2 weeks, beginning 2 days prior to giving birth. This antibiotic treatment resulted in ~4-log depletion of culturable bacteria in the oral cavity of nursing mothers and ~3-log depletion in the skin of newborn mice (Figure 4A). Furthermore, antibiotic treatment resulted in robust reduction of skin bacteria in neonatal mice as assessed by quantitative PCR of eubacteria 16S ribosomal RNA (rRNA) DNA (Figure 4B) and did not alter the number of MCs in the skin (Figure S3). Importantly, treatment with antibiotics led to reduced disease incidence and score compared to untreated Nlrp3^{R258W} mice (Figure 4C; Figure S4). Furthermore, antibiotic-treated Nlrp3^{R258W} mice had less weight loss (Figure 4D) and splenomegaly (Figure 4E) than their untreated littermates. Furthermore, administration of antibiotics led to reduced amounts of IL-1 β in the serum and skin of Nlrp3^{R258W} mice compared to untreated Nlrp3^{R258W} mice (Figures 4F and 4G). Notably, antibiotic treatment also reduced the amounts of TNF- α in the skin of both wild-type and Nlrp3^{R258W} littermates (Figure 4G). However, the production of TNF- α was comparable in the skin of untreated wild-type and Nlrp3^{R258W} littermates (Figure 4G). If the microbiota induces TNF- α to trigger skin disease in neonatal Nlrp3^{R258W} mice, administration of TNF- α or induced release of endogenous TNF- α release would be expected to trigger skin disease in antibiotic-treated Nlrp3^{R258W} mice. Notably, intradermal administration of compound 48/80, a molecule that induces the release of TNF- α , and other molecules from connective-tissue-type MC secretory granules (Mousli et al., 1990) or intradermal injection of recombinant (r) TNF- α induced skin disease (Figure 4H) and IL-1 β production (Figure 4I) in antibiotic-treated Nlrp3^{R258W} mice, but not in wild-type littermates.

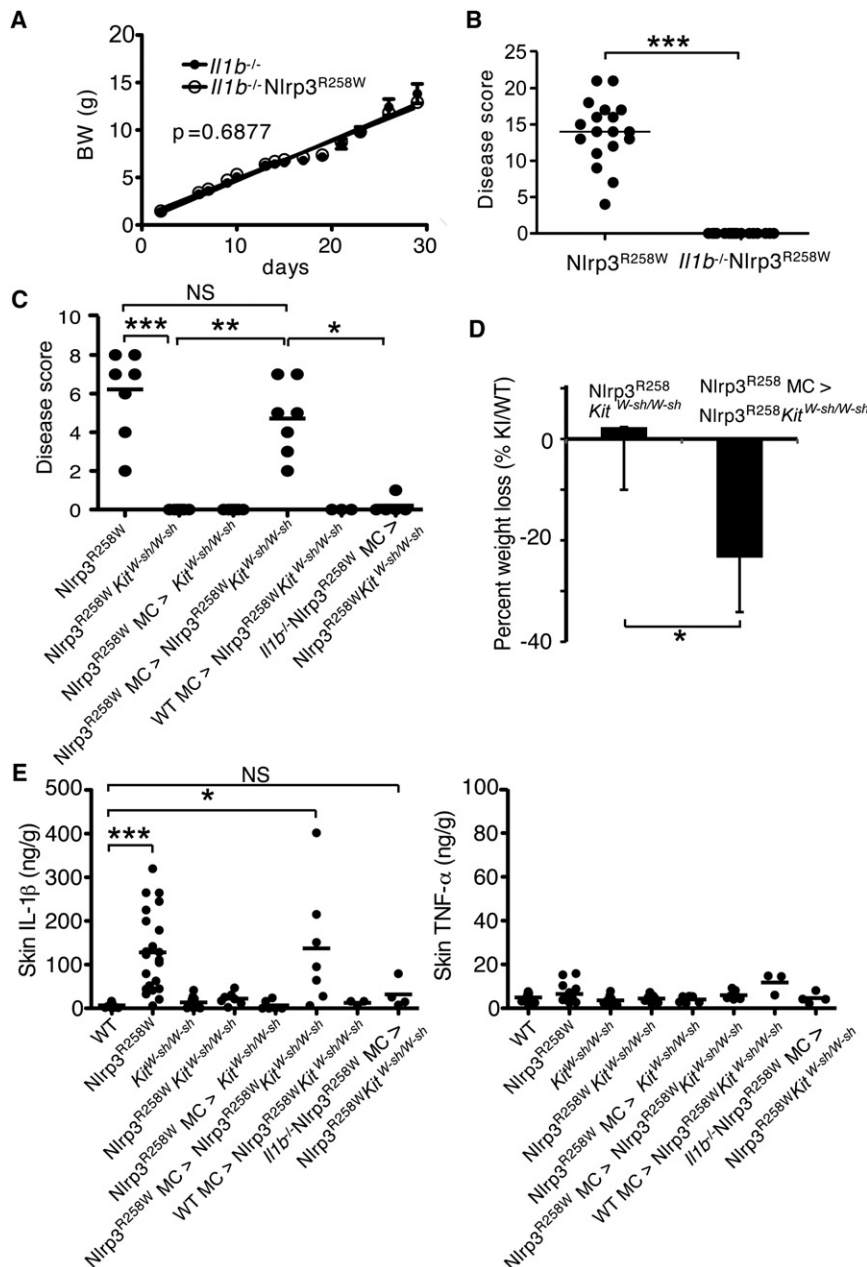


Figure 3. IL-1 β Production by MCs Expressing the *Nlrp3^{R258W}* Mutation Is Important for Skin Disease in *Nlrp3^{R258W}* Mice

(A) Linear growth curve of *Nlrp3^{R258W}/Il1b^{-/-}* (n = 6) and *Il1b^{-/-}* littermates (n = 8).

(B) Skin-disease score from 2-week-old *Nlrp3^{R258W}* and *Nlrp3^{R258W}/Il1b^{-/-}* mice. Dots represent individual mice. Bar represents mean values. *** $p < 0.001$.

(C) Skin-disease score in *Nlrp3^{R258W}*, *Nlrp3^{R258W}/Kit^{W-sh}/Kit^{W-sh}*, and indicated recipient mice reconstituted in the skin with MCs from indicated mice at PND 1. Dots represent individual mice. Bar represents mean values.

(D) Percentage of weight loss of *Nlrp3^{R258W}* *Kit^{W-sh}/Kit^{W-sh}* mice and *Nlrp3^{R258W}/Kit^{W-sh}/Kit^{W-sh}* mice reconstituted with MCs from *Nlrp3^{R258W}* mice at PND 1. Results shown are from 2-week-old mice and normalized to weight of control B6 *Kit^{W-sh}/Kit^{W-sh}* littermates (WT). * $p < 0.05$, KI, knockin.

(E) IL-1 β (left panel) and TNF- α (right panel) levels in skin of *Nlrp3^{R258W}*, *Nlrp3^{R258W}/Kit^{W-sh}/Kit^{W-sh}*, and indicated recipient mice reconstituted in the skin with MCs from indicated mice at PND 1. Results are from 2-week-old mice. Bars represent mean values.

Significance in (D) and (E) was determined by one-way ANOVA; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. NS, not significant. Data shown are representative of two independent experiments (C)–(E).

These results indicate that the indigenous microbiota contribute to TNF- α and IL-1 β production and the development of skin disease in newborn *Nlrp3^{R258W}* mice.

Neutralization of TNF- α Abrogates the Development of Skin Disease in Neonatal *Nlrp3^{R258W}* Mice

Because treatment with antibiotics reduced the amounts of TNF- α in the skin, and TNF- α alone can induce IL-1 β secretion in MCs from *Nlrp3^{R258W}* mice (Figures 4G and 2C), we tested whether TNF- α plays a role in the development of skin disease in newborn *Nlrp3^{R258W}* mice. To assess this, we treated *Nlrp3^{R258W}* mice at PND 1 with TNF- α -blocking monoclonal antibody or isotype-matched control immunoglobulin G (IgG) intra-

peritoneally. Neutralization of TNF- α abrogated the loss of body weight, the development of skin disease, and splenomegaly in newborn *Nlrp3^{R258W}* mice (Figures 5A, 5B and 5C; Figure S5A). Histological analysis revealed that administration of TNF- α antibody prevented inflammatory skin disease in newborn *Nlrp3^{R258W}* mice (Figure 5D). Notably, treatment with TNF- α antibody inhibited the production of IL-1 β in *Nlrp3^{R258W}* mice (Figure 5E). After weaning, cutaneous disease gradually disappeared in *Nlrp3^{R258W}* mice, but as previously reported (Meng et al., 2009), a significant number of adult *Nlrp3^{R258W}* mice relapsed at older age (>12 weeks) and developed skin disease affecting the ears, the top of the head, and the tail base region (Figure S5B). In contrast to neonatal mice, treatment with TNF- α antibody did not inhibit ongoing skin disease in adult *Nlrp3^{R258W}* mice (Figure 5F). Collectively, these results indicate that TNF- α is critical for the development of disease in neonatal mice, but its inhibition does not improve disease in adult *Nlrp3^{R258W}* mice.

MC-Intrinsic TNF- α , but Not IL-1 β , Is Critical for Skin Disease Induced by Compound 48/40 in Adult *Nlrp3^{R258W}* Mice

TNF- α is a key cytokine that is stored in the granules of MCs (Echtenacher et al., 1996; Gordon and Galli, 1990; Maurer

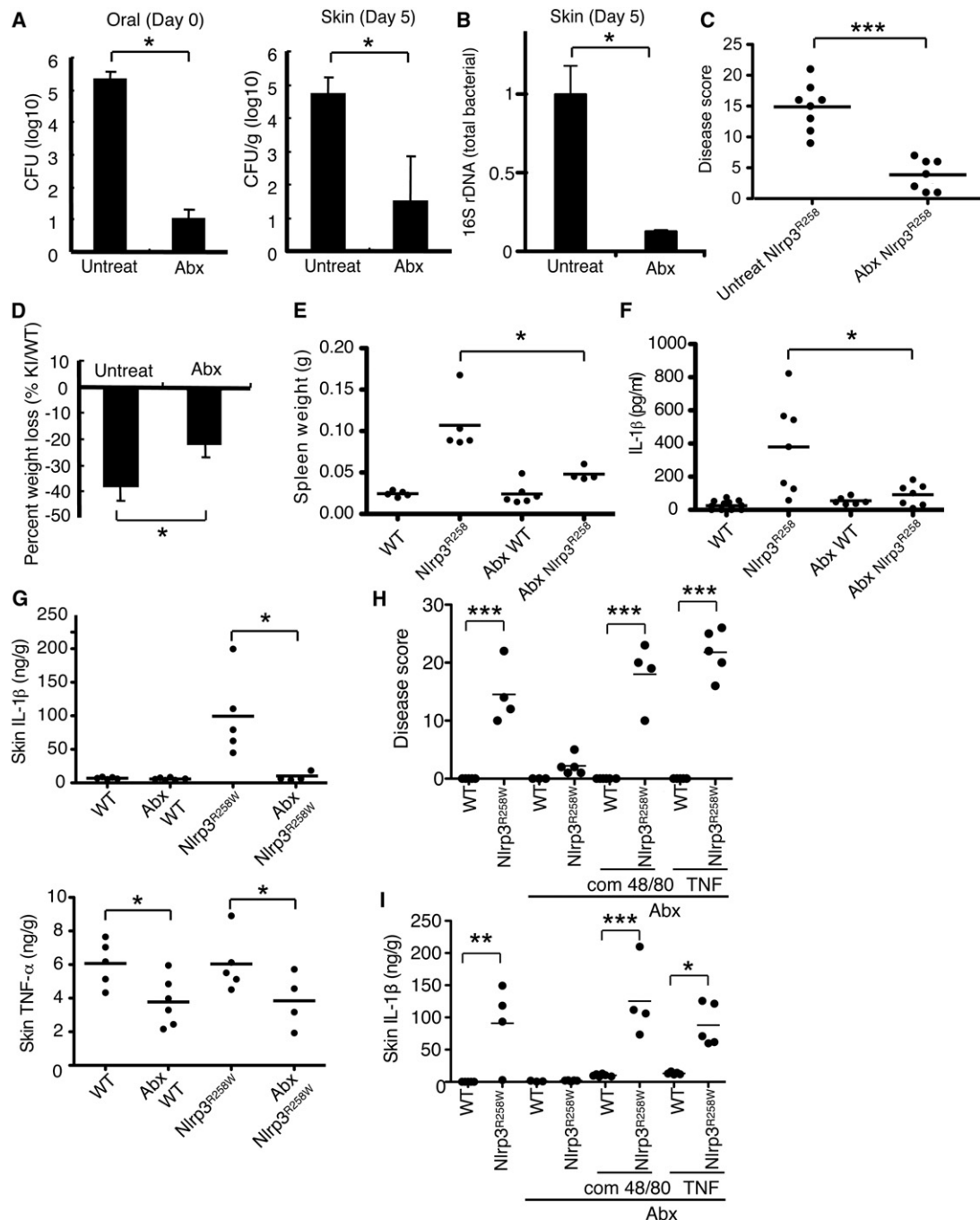


Figure 4. Depletion of the Microbiota Inhibits Disease Development in Nlrp3^{R258W} Mice

(A) Number of culturable bacteria in the oral cavity of nursing female mice (left panel) and skin (posterior collar region) of newborn mice (right panel) in untreated (Untreat) and antibiotic-treated (Abx) mice. Results are mean \pm SD (n = 5).

(B) Normalized 16S rDNA gene analysis of the skin from untreated and Abx-treated mice.

(C) Skin-disease score in untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice. Dots represent individual mice. Bar represents mean values.

(D) Percentage of weight loss in untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice. Results shown are normalized to weight of control B6 littermates (WT). *p < 0.05. KI, knockin.

(E) Spleen weight of untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice and WT mice.

(F) and (G) IL-1β in serum (F) and skin (G, top panel) and TNF-α in skin (G, bottom panel) of untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice and wild-type littermates. Each dot represents an individual mouse. Horizontal bars indicate mean values.

(H) and (I) Skin-disease score (H) and skin IL-1β (I) in untreated and Abx-treated 2-week-old Nlrp3^{R258W} mice induced by compound 48/80 or TNF-α intradermal injection. Dots represent individual mice. Bar represents mean values. (I) *p < 0.05, **p < 0.01, ***p < 0.001. Data shown are representative of two independent experiments.

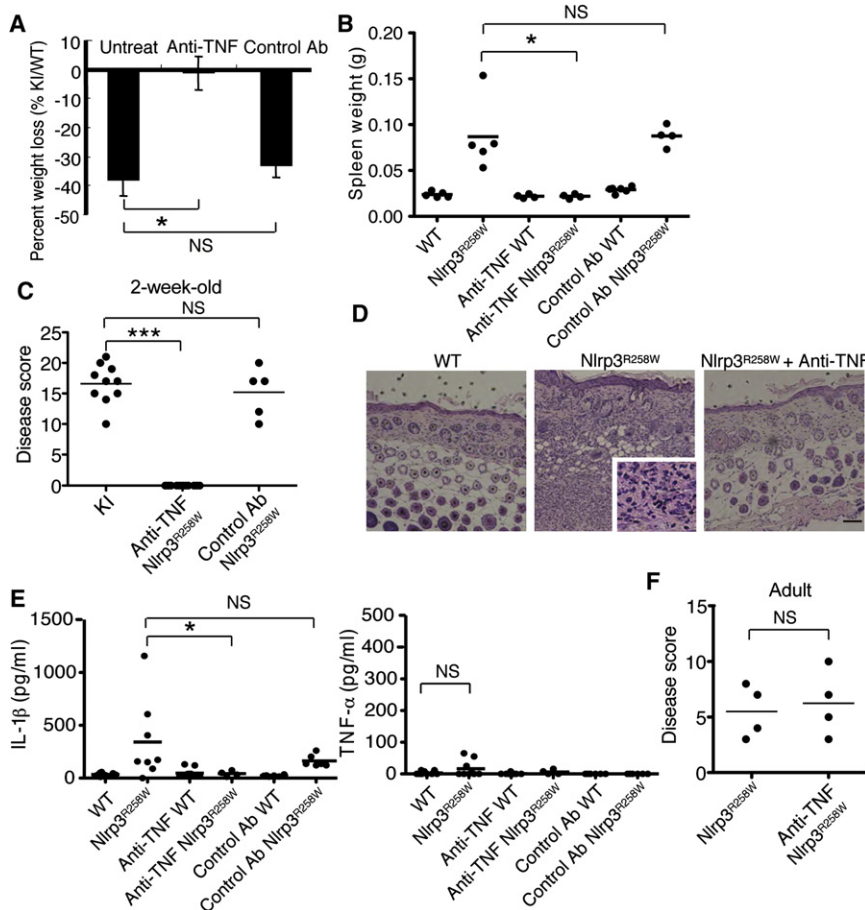


Figure 5. Neutralization of TNF- α Abrogates the Development of Skin Disease in Neonatal Nlrp3^{R258W} Mice

(A) Percentage of weight loss in untreated and Nlrp3^{R258W} mice treated with TNF- α antibody or control antibody at PND 1. Results shown are from 2-week-old mice normalized to the weight of control B6 littermates (WT). * $p < 0.05$. KI, knockin. (B) Spleen weight of 2-week-old untreated mice and antibody-treated Nlrp3^{R258W} and WT littermates. Mice were treated with control or TNF- α antibody at PND 1. * $p < 0.05$. NS, not significant. (C) Skin-disease score in 2-week-old untreated and antibody-treated Nlrp3^{R258W} littermates. Mice were treated with control or TNF- α antibody at PND 1. Dots represent individual mice. Bar represents mean values. *** $p < 0.001$. NS, not significant. (D) Representative H&E staining of skin from 2-week-old untreated WT and Nlrp3^{R258W} littermates and a littermate treated with TNF- α antibody. (E) Serum IL-1 β (left panel) and TNF- α (right panel) levels in 2-week-old untreated WT and Nlrp3^{R258W} mice and antibody-treated littermates. Mice were treated with control or TNF- α antibody at PND 1. (F) Skin-disease score in adult untreated and antibody-treated Nlrp3^{R258W} mice. Mice were treated with TNF- α antibody twice weekly. Horizontal bars indicate mean values. Dots represent individual mice. NS, not significant. Data shown are representative of three (A–D) and two (E and F) independent experiments.

et al., 2006; Suto et al., 2006). Therefore, we hypothesized that granule-associated TNF- α within MCs may be important in triggering skin disease in Nlrp3^{R258W} mice. To test this, we injected compound 48/80 into the uninvolved skin of wild-type, Nlrp3^{R258W}, and Nlrp3^{R258W} *Kit*^{W-sh/W-sh} adult mice and assessed disease and IL-1 β production in the skin. Administration of compound 48/80 induced marked neutrophilic inflammation in the skin and IL-1 β cytokine production at the injection site in Nlrp3^{R258W} mice, but not in wild-type or Nlrp3^{R258W} *Kit*^{W-sh/W-sh} mice (Figures 6A and 6B; Figure S6). To determine whether TNF- α and/or IL-1 β produced by MCs is important for inducing skin inflammation in Nlrp3^{R258W} mice, we adoptively transferred BMCMCs from wild-type, *Tnfa*^{-/-}, and *Il1b*^{-/-} mice into the skin of adult Nlrp3^{R258W} *Kit*^{W-sh/W-sh} mice. MC-deficient Nlrp3^{R258W} *Kit*^{W-sh/W-sh} mice reconstituted with MCs from wild-type, but not *Tnfa*^{-/-} mice, developed neutrophilic skin inflammation and increased IL-1 β production upon administration of compound 48/80 at the site of injection (Figures 6A and 6B). Notably, reconstitution with BMCMCs from *Il1b*^{-/-} mice induced inflammation and IL-1 β production (Figures 6A and 6B). These results indicate that TNF- α from MCs can induce IL-1 β production and inflammatory skin disease in Nlrp3^{R258W} mice. Furthermore, MC-intrinsic IL-1 β is not required for 48/80-induced skin disease and IL-1 β production in adult Nlrp3^{R258W} mice.

Administration of TNF- α Induces IL-1 β -Dependent Skin Inflammation in Adult Nlrp3^{R258W} Mice

We next asked whether TNF- α is sufficient to induce skin inflammation via IL-1 β in adult Nlrp3^{R258W} mice. To test this, we first injected rTNF- α into the skin of the ear pinna of disease-free adult Nlrp3^{R258W} and wild-type mice. Administration of rTNF- α induced marked thickening and redness in the skin of Nlrp3^{R258W} B6 mice, but not wild-type mice (Figure S7). Furthermore, skin disease induced by rTNF- α was reduced in Nlrp3^{R258W} *Kit*^{W-sh/W-sh} and abrogated in Nlrp3^{R258W} mice lacking IL-1 β (Figure S7). Histological examination revealed marked neutrophil infiltration in the dermis of Nlrp3^{R258W} mice injected with rTNF- α , which was abolished in Nlrp3^{R258W} *Il1b*^{-/-} mice (Figure 7A). Consistent with these findings, the amounts of IL-1 β in the skin of Nlrp3^{R258W} increased at the site of TNF- α administration, and this was also observed in Nlrp3^{R258W} *Kit*^{W-sh/W-sh} mice (Figure 7B). Taken together, these results indicate that TNF- α can trigger skin inflammation via IL-1 β in adult Nlrp3^{R258W} mice and that this is largely independent of MCs.

DISCUSSION

CAPS-associated missense *NLRP3* mutations result in enhanced activation of caspase-1 and secretion of IL-1 β , leading to inflammatory disease in the skin and other organs. However,

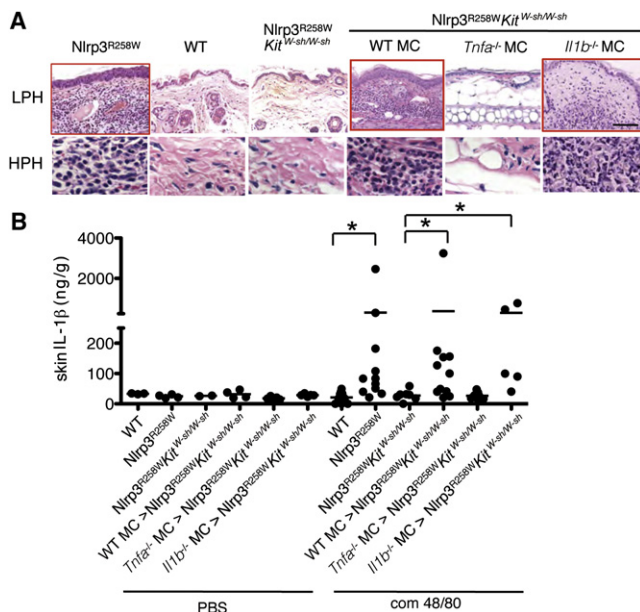


Figure 6. TNF- α Produced by MCs Can Induce Skin Disease in Nlrp3^{R258W} Mice

(A) Adult Nlrp3^{R258W}, B6 (WT), Kit^{W-sh/W-sh}, and Nlrp3^{R258W} Kit^{W-sh/W-sh} mice reconstituted with MCs from WT, Tnfa^{-/-}, and Il1b^{-/-} mice. Mice were intradermally injected with compound 48/80 at the site of reconstitution. Representative images of low power histology (LPH) and high power histology (HPH) of H&E stained skin are shown. Bar indicates 100 μ m.

(B) IL-1 β levels in skin of wild-type (WT), Nlrp3^{R258W} Kit^{W-sh/W-sh}, and Nlrp3^{R258W} Kit^{W-sh/W-sh} mice reconstituted with MCs from WT, Tnfa^{-/-}, and Il1b^{-/-} mice. Mice were injected intradermally with compound 48/80 at the site of MC reconstitution. Bars represent mean values. Significance was determined by one-way ANOVA; *p < 0.05. NS, not significant. Data shown are representative of two independent experiments.

the cells and the mechanism that orchestrate NLRP3-dependent IL-1 β -driven inflammatory disease in vivo remain unclear. We show here that MCs play a critical role in the initiation of skin inflammation and systemic disease in neonatal Nlrp3^{R258W} mice. Reconstitution experiments revealed that production of IL-1 β by MCs expressing the Nlrp3 mutation was important for skin disease and delayed growth in neonatal mutant mice. Our studies suggest that colonization of newborn mice by the microbiota induces local production of TNF- α , which primes MCs to elicit dysregulated production of IL-1 β , causing skin and systemic disease. This model is consistent with the observation that MCs expressing the Nlrp3^{R258W} mutation require stimulation with TNF- α or LPS to secrete IL-1 β . Unlike in normal cells, this priming step is sufficient to trigger caspase-1 activation and IL-1 β secretion by inducing the expression of Nlrp3 and pro-IL-1 β (Bauernfeind et al., 2009; Franchi et al., 2009b). In our Nlrp3^{R258W} model, the microbiota are critical to induce IL-1 β -driven disease in neonatal mice. There is evidence that environmental factors may play a role in triggering CAPS in humans. For example, inflammatory episodes in FCAS are often induced by exposure to cold, whereas NOMID patients are born healthy, but they develop disease in early infancy. Thus, it is possible that exposure to cold, which induces cytokines or other proinflammatory molecules (Theoharides et al., 2004; Zhu et al., 1996),

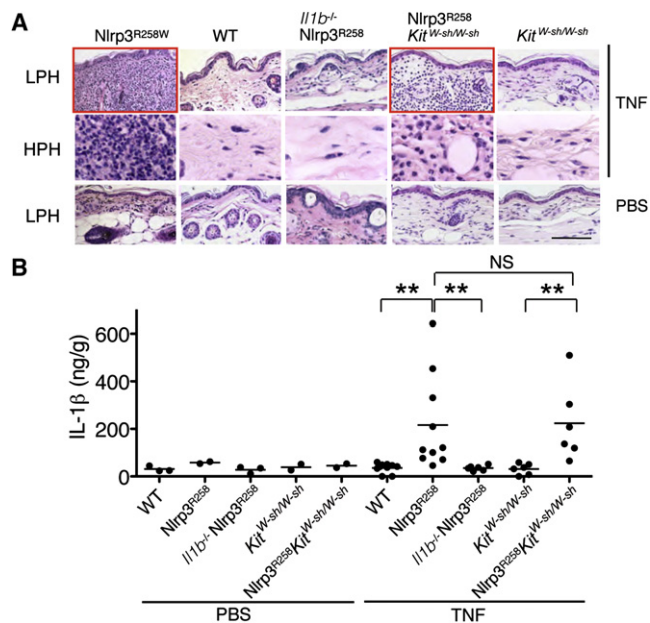


Figure 7. TNF- α Administration Induces IL-1 β -Dependent Skin Inflammation in Nlrp3^{R258W} Mice

(A) Adult Nlrp3^{R258W}, B6 (WT), Nlrp3^{R258W} Il1b^{-/-}, Nlrp3^{R258W} Kit^{W-sh/W-sh}, and B6-Kit^{W-sh/W-sh} mice were injected intradermally with TNF- α or PBS in the ear pinna. Representative images of low power histology (LPH) and high power histology (HPH) of H&E stained skin 48 hr after injection are shown. Bar indicates 100 μ m.

(B) IL-1 β levels in the skin of indicated mice injected intradermally with PBS or TNF- α in the ear pinna. Results shown are from 48 hr after injection. Bars represent mean values. Significance was determined by one-way ANOVA; **p < 0.01. NS, not significant. Data shown are representative of two independent experiments.

can prime MCs to elicit IL-1 β -driven disease. Similarly, colonization by microbes could trigger inflammatory disease in NOMID patients. Clearly, further work is needed to understand how environmental factors contribute to the pathogenesis of CAPS in humans.

MCs are widely distributed throughout the body; they are located near epithelial surfaces such as the skin and airways that are normally exposed to environmental cues (Galli et al., 2008). Unlike macrophages, MCs can secrete a wide array of granule-associated, preformed biologically active products, including TNF- α , in response to a variety of stimuli, including microbes (Gordon and Galli, 1990). Hence, exposure to microbial products from commensal organisms could induce exocytosis of MCs and secretion of TNF- α to prime mutant MCs for IL-1 β production in an autocrine and paracrine manner. Alternatively, newly synthesized TNF- α derived from stimulation of MCs by microbial molecules could mediate priming of MCs. Consistent with the former model, administration of 48/80, a compound that induces MC exocytosis, triggered IL-1 β production and neutrophilic inflammation in the skin of Nlrp3^{R258W} mice, but not in Nlrp3^{R258W} mice deficient in MCs or in wild-type mice. Furthermore, reconstitution of MC-deficient Nlrp3^{R258W} mice with MCs from wild-type mice, but not Tnfa^{-/-} mice, induced IL-1 β production and skin inflammation at the site of compound

48/80 administration. Other key findings also support an important role for TNF- α in initiating skin disease in Nlrp3^{R258W} mice. First, neutralization of endogenous TNF- α abrogated the development of skin disease. Second, intradermal injection of TNF- α triggered local IL-1 β production and inflammatory skin disease, which was blocked in the absence of IL-1 β , specifically in adult Nlrp3^{R258W} mice. In adult Nlrp3^{R258W} mice, IL-1 β production induced by administration of TNF- α was undisturbed in the absence of MCs. Similarly, MC-intrinsic IL-1 β production was dispensable for skin disease triggered by intradermal administration of compound 48/80 into adult Nlrp3^{R258W} mice. Thus, IL-1 β from a cell source other than MCs is important for skin disease induced by 48/80 or rTNF administration in Nlrp3^{R258W} mice. In these adult-mouse models, MC-derived TNF- α is likely to act on an intermediary myeloid cell-type that is the source of IL-1 β to cause disease. A role for cells other than MCs in skin disease is also supported by the finding that adoptive transfer of MCs expressing the Nlrp3^{R258W} mutation induced skin disease in MC-deficient Nlrp3^{R258W} *Kit*^{W-sh/W-sh} mice, but not in B6-*Kit*^{W-sh/W-sh} mice. Collectively, the results indicate that resident MCs expressing CAPS-associated NLRP3 mutations produce TNF- α locally upon exposure to commensals, leading to IL-1 β secretion, which in turn primes additional innate immune cells expressing the NLRP3 mutation to amplify local IL-1 β production and inflammatory disease. Although production of both TNF- α and IL-1 β by MCs is important for inducing disease in the neonatal model, MC-independent production of IL-1 β appears to be critical for disease in adult Nlrp3^{R258W} mice. Although additional studies are needed to identify the cell(s) involved, it is likely that macrophages, dendritic cells, or neutrophils expressing mutant Nlrp3 could contribute to IL-1 β production and the development of disease.

Activation of the Nlrp3 inflammasome leads not only to IL-1 β secretion, but also to production of IL-18, whose maturation is mediated by caspase-1 (Arend et al., 2008). In addition, monocytes from CAPS patients carrying disease-associated NLRP3 mutations exhibit enhanced pyroptosis after stimulation with LPS (Fujisawa et al., 2007; Willingham et al., 2007). Therefore, it is possible that activities other than those induced via IL-1 β contribute to inflammatory disease in CAPS patients. Consistent with this possibility, genetic deletion of IL-1 signaling was associated with marked improvement, but it did not abrogate disease in mice harboring the disease-associated A350V or L351P Nlrp3 mutations (Brydges et al., 2009). In contrast, we found that deletion of IL-1 β in Nlrp3^{R258W} mice fully rescued the mutant mice from disease. In mice, the A350V or L351P Nlrp3 mutations confer a more severe phenotype than the R258W mutation, and this could explain, at least in part, the difference in results (Brydges et al., 2009; Meng et al., 2009). In the last decade, biological inhibitors of IL-1 signaling or IL-1 β antibodies have become the most effective treatments for CAPS patients (Goldbach-Mansky et al., 2006; Hoffman et al., 2008; Lachmann et al., 2009). In contrast, anti-TNF- α treatment had some positive effects; however, its efficacy was very limited compared to anti-IL-1 β therapies (Ebrahimi-Fakhari et al., 2010; Federico et al., 2003; Gunduz et al., 2008; Kallinich et al., 2005; Matsubara et al., 2006). In accordance with the human studies, neutralization of IL-1 β with antibody was very effective in reducing inflammatory disease in adult Nlrp3^{R258W} mice (Meng et al., 2009). In

contrast, TNF- α -blocking antibody abrogated the development of disease in neonatal Nlrp3^{R258W} mice, whereas it was ineffective in reducing ongoing disease in adult Nlrp3^{R258W} mice, which is consistent with studies in CAPS patients. These results suggest that TNF- α is important in the initiation phase of the disease, but it plays a minimal or no role at the later phases of the disease. Although further work is needed to understand the role of TNF- α in the pathogenesis of CAPS, one possibility is that TNF- α is critical for the initial induction of pathogenic IL-1 β . Once IL-1 β is produced, MCs and other IL-1 β -producing cells expressing mutant Nlrp3 can rely on IL-1 β for priming and eliciting IL-1 β -driven inflammatory disease. The latter could explain why therapies targeting IL-1 β are very effective in treating CAPS patients with disease, whereas anti-TNF- α blockade is not. In addition to CAPS, another IL-1 β -driven autoinflammatory disease, familial Mediterranean fever (FMF), is associated with excess production of IL-1 β through NLRP3-independent activation of caspase-1 (Chae et al., 2006; Papin et al., 2007). A recent study demonstrated that FMF-associated pyrin mutant mice also developed skin inflammation at as early as 1 week of age and suffered from severe inflammation in multiple tissues (Chae et al., 2011). Because the time course and disease phenotype of pyrin mutant mice are similar to those of Nlrp3 mutant mice, it is possible that MC-mediated mechanisms observed in Nlrp3^{R258W} mice also contribute to the development of disease in pyrin mutant mice and related IL-1 β -driven diseases in humans.

EXPERIMENTAL PROCEDURES

Mice

Nlrp3^{R258W} mice have been described (Meng et al., 2009). C57BL/6, C57BL/6-*Kit*^{W-sh/W-sh} (B6.CG-*Kit*^{W-sh}/HNIhrJaeBsmJ), and *Tnfa*^{-/-} (B6.129S6-*Tnfa*^{tm1Gkl/J}) mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). B6.11b^{-/-} mice were originally obtained from Y. Iwakura (University of Tokyo, Tokyo). All strains were housed under pathogen-free conditions. The animal studies were conducted under approved protocols by the University of Michigan Committee on Use and Care of Animals.

Preparation and Adoptive Transfer of Mast Cells

The preparation of BMCMCs was previously described (Yamada et al., 2003). The purity of MCs was > 95% based on toluidine blue staining and surface expression of CD117 and Fc ϵ R.

Adoptive transfer of MCs into MC-deficient *Kit*^{W-sh/W-sh} mice was performed as described (Grimbaldeston et al., 2005). Briefly, BMCMCs were obtained after a 4–5 week culture of bone marrow cells in medium containing 20 ng/ml recombinant mouse IL-3 (R&D Systems). For MC-reconstitution studies, BMCMCs were adoptively transferred via intradermal injection of 2×10^6 cells in 40 μ l PBS into the ear pinna (at 4–8 weeks old) or posterior collar area (at PND 1) of MC-deficient recipient mice. Mice were evaluated at 2–3 weeks (neonatal mice) or 4–5 weeks (adult mice) after intradermal transfer of BMCMCs.

Administration of TNF- α Antibody and Antibiotics

Anti-TNF- α -blocking IgG (rat clone; MPG-XT3) was a gift from T. Moore (University of Michigan). Newborn mice were given 60 μ g in 30 μ l of TNF- α antibody or isotype-matched control rat IgG (Sigma-Aldrich), intraperitoneally. Adult Nlrp3^{R258W} mice were treated with 500 μ g of TNF- α antibody intraperitoneally, weekly for 2 weeks. For antibiotic treatment, pregnant mice received an antibiotic cocktail (1 g/L of ampicillin, 0.5 g/L of vancomycin, 1 g/L of metronidazole, and 1 g/L of neomycin) in the drinking water 2 days before delivery, and the treatment continued for 2 weeks. Newborn mice were given 50 μ g in 40 μ l of rTNF- α at PND 3 or 1 μ g in 40 μ l of compound 48/80 at PND 3 and PND 6,

intradermally. Bacterial depletion was assessed in the mothers' oral cavity and newborns' skin through collection and homogenization of tissue in sterile PBS. The number of bacteria was determined by serial dilution of tissue samples, followed by serial plating on brain-heart infusion broth for 48 hr at 37°C under aerobic conditions.

Skin-Disease Score

The severity of skin disease was evaluated using a modified skin-index scoring system (Matsuda et al., 1997). Briefly, skin inflammation was evaluated every other day as a cumulative score. The total disease-severity score from three areas (head and neck, body, and perianal region) was defined as the sum of the individual scores, graded as 0 (none), 1 (mild), 2 (moderate), and 3 (severe) for each of four disease signs (erythema, alopecia, scales, and erosions). The maximum score was 36.

Intradermal Injection

Adult mice (6–12 weeks old) were given compound 48/80 (1 µg in 20 µl per ear pinna; Sigma-Aldrich), rTNF-α (500 ng in 20 µl per ear pinna; PeproTech), or control PBS via intradermal injection. Tissue samples were collected 48 hr after injection for analysis.

Histology

Skin tissue was formalin fixed, paraffin embedded, and sectioned for hematoxylin and eosin (H&E), toluidine blue, and safranin O staining. For immunofluorescence staining, sections were subjected to labeling with anti-IL-1β (Armenian Hamster clone B122; Leinco Technologies) followed by fluorescein isothiocyanate-conjugated secondary antibodies and Texas red-Avidin staining.

Immunoblotting

Cells were lysed together with the cell supernatant by the addition of 1% NP-40 complete protease inhibitor cocktail (Roche) and 2 mM dithiothreitol. Clarified lysates were resolved by SDS-PAGE and transferred to polyvinylidene fluoride membranes by electroblotting. The rabbit anti-mouse caspase-1 was a kind gift from P. Vandanaabele (Ghent University, Belgium).

Preparation of Skin Extracts

Skin tissue (5 × 5 mm area) was removed and homogenized as illustrated in Figure S2A. The skin homogenates were centrifuged and supernatants were collected for cytokine measurements by ELISA.

Cytokine Levels

Chemokines and cytokines were measured with ELISA kits (R&D Systems).

Statistical Analysis

Most of the data were compared by two-tailed t test with unequal variance (GraphPad Prism). Analysis of data involving multiple comparisons was performed by one-way ANOVA. The slope of two regression lines was compared using GraphPad Prism. Differences were considered significant when p values were less than 0.05.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2012.04.013>.

ACKNOWLEDGMENTS

We thank S. Koonse for animal husbandry, J. Whitfield for ELISAs, and Y. Iwakura for the generous gift of mice. This work was supported by NIH grants R01AR059688 and R01AI06331, and funds to the Michigan Comprehensive Cancer Center Immunology Monitoring Core from the University of Michigan's Cancer Center Support Grant. Y.N. was supported by fellowships from Chiba University Global COE Program, the Cell Science Research Foundation, and the Kanae Foundation for the Promotion of Medical Science.

Received: December 26, 2011

Revised: March 20, 2012

Accepted: April 17, 2012

Published online: July 19, 2012

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